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Untangling reticulate evolutionary relationships among New World and Hawaiian mints (Stachydeae, Lamiaceae) ☆

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ABSTRACT

The phenomenon of polyploidy and hybridization usually results in novel genetic combinations, leading to complex, reticulate evolution and incongruence among gene trees, which in turn may show different phylogenetic histories than the inherent species tree. The largest tribe within the subfamily Lamioideae (Lamiaceae), Stachydeae, which includes the globally distributed *Stachys*, and one of the largest Hawaiian angiosperm radiations, the endemic mints, is a widespread and taxonomically challenging lineage displaying a wide spectrum of morphological and chromosomal diversity. Previous molecular phylogenetic studies have showed that while the Hawaiian mints group with Mexican–South American *Stachys* based on chloroplast DNA sequence data, nuclear ribosomal DNA (nrDNA) sequences suggest that they are most closely related to temperate North American *Stachys*. Here, we have utilized five independently inherited, low-copy nuclear loci, and a variety of phylogenetic methods, including multi-locus coalescence-based tree reconstructions, to provide insight into the complex origins and evolutionary relationships between the New World *Stachys* and the Hawaiian mints. Our results demonstrate incongruence between individual gene trees, grouping the Hawaiian mints with both temperate North American and Meso-South American *Stachys* clades. However, our multi-locus coalescence tree is concurrent with previous nrDNA results placing them within the temperate North American *Stachys* clade. Our results point toward a possible allopolyploid hybrid origin of the Hawaiian mints arising from temperate North American and Meso-South American ancestors, as well as a reticulate origin for South American *Stachys*. As such, our study is another significant step toward further understanding the putative parentage and the potential influence of hybridization and incomplete lineage sorting in giving rise to this insular plant lineage, which following colonization underwent rapid morphological and ecological diversification.

1. Introduction

1.1. Natural hybridization, polyploidy and factors affecting species relationships

Natural hybridizations have had far-reaching effects in angiosperm evolution, and it has been estimated that approximately 25% of vascular plants hybridize with other plant species and about 11% of plant species are products of hybridization events (Ellstrand et al., 1996; Mallet, 2007). Changes in ploidy levels are the most common effect of hybridization, mostly resulting from the union of unreduced gametes from two parental species (Soltis and Soltis, 2000; Husband, 2004). The novel genetic combinations in polyploid genomes lead to innovative genetic changes, which often give rise to new species and other forms of organismal diversity (Mallet, 2007; Van de Peer et al., 2009) and may affect their colonization and dispersal abilities allowing them to occupy environmental niches unavailable to their parents (Soltis and Soltis, 2000; Bento et al., 2008).

Hybrid progeny with genetic material displaying a mosaic of phylogenetic signals may lead to incongruence between different gene trees thereby confounding phylogenetic reconstructions. However, incongruence can also arise as a result of stochastic or population-level events, including incomplete lineage sorting, non-homologous sampling of duplicated genes leading to misinterpretations between orthologs and paralogs, and horizontal gene transfer (Álvarez and Wendel, 2003; Maddison, 1997), causing individual gene trees to differ from the underlying species tree (McBreen and Lockhart, 2006; Holland et al., 2008). Reticulate

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evolution is well documented among plants throughout their genealogical histories (Rieseberg, 1997; Mallet, 2007), and phylogenetic incongruence within plant genomes has been observed in numerous molecular studies (Pirie et al., 2008; Vargas et al., 2009). Thus, reconstruction of species relationships in polyploid groups with a high degree of reticulation may be a challenging task. In such instances, adding larger numbers of markers does not necessarily lead to a better resolution of the species trees, but instead the study of different kinds of markers can shed further light into the evolutionary histories of such species complexes (Small et al., 2004; Hughes et al., 2006; Rousseau-Gueutin et al., 2009).

1.2. Low-copy nuclear loci in the study of hybridization history

Historically, plant molecular phylogenetics has depended to a great extent on data generated from plastid (cpDNA) and nuclear ribosomal (nrDNA) DNA sequences, since their high copy number facilitates their utility (Álvarez and Wendel, 2003; Shaw et al., 2005). However, the uniparental inheritance of cpDNA masks evidence of reticulation in hybridizing species, and nrDNA undergoes concerted evolution due to gene conversion and unequal crossing over, leading to reduction in variation, lack of sufficient resolution for phylogenetic inferences, and masking of hybridized histories. In such cases, single or low-copy nuclear genes are able to compensate for some of the problems encountered with cpDNA and nrDNA markers (Sang, 2002; Small et al., 2004; Curto et al., 2012). Single- or low-copy nuclear genes are biparental, independently inherited, and exhibit less concerted evolution leading to a greater conservation of gene homeologs from ancestral genomes, providing evolutionary signatures of unique gene copies tracing hybrid reticulate history within lineages (Mort and Crawford, 2004; Duarte et al., 2010). Introns from low-copy markers may provide higher variability than coding regions, due to a higher rate of nucleotide substitutions in the former, and synonymous positions not being conserved in the latter group (Sang, 2002), thereby presenting the possibility of being phylogenetically more informative in closely related species where unambiguous alignments can be performed. Low-copy nuclear markers have been successfully used in previous studies to reconstruct allopolyploid origins in plants (Brysting et al., 2007; Rousseau-Gueutin et al., 2009; Liu et al., 2011). Despite numerous positive attributes, low-copy genes are not completely devoid of drawbacks, including paralogy, and population genetic processes, such as incomplete lineage sorting, in which cases the individual gene trees may not resemble the actual species tree, obfuscating actual phylogenetic relationships. However, these drawbacks can be overcome when combined with cloning, wherein the utility of low-copy nuclear genes can aid in the difficulties involved in direct reconstruction of reticulate evolution. By cloning the homoeologous loci derived from both parents, an allotetraploid genome can be separated into two independent units, with each homoeolog tracing its own parental lineage. Phylogenetic reconstructions from the cloned homoeologs together with the genes of the putative parents may aid the reconstruction of the diverged histories of the parental lineages from reticulate evolutionary history (Sang and Zhong, 2000).

1.3. New World Stachys, Hawaiian endemic mints and our current study

Stachydeae, the largest tribe of the subfamily Lamioideae (Lamiaceae), consists of about 470 species, has a globally widespread distribution, and exhibits considerable chromosomal diversity (Scheen et al., 2010; Bendiksby et al., 2011; Salmaki et al., 2013). Stachydeae is comprised of three endemic Hawaiian genera and at least seven Old World (OW) genera (e.g., *Sideritis*, *Prasium*, *Chamaesphacos*, *Suzukia*, and *Thuspeinanta*) that are nested within the largest genus *Stachys*, making this genus paraphyletic (Lindqvist and Albert, 2002). *Stachys* itself has a worldwide distribution, with the New World (NW) *Stachys* (Fig. 1) presenting a particularly challenging yet interesting system for phylogenetic reconstructions due to its wide range of morphological and cytological features and the inclusion of one of the largest plant lineages in the Hawaiian Islands, the Hawaiian endemic mints. The chromosome numbers for this Stachydeae lineage vary between $2n = 34$ or 68 in species with a predominant eastern or widespread North American (NA) distribution and $2n = 64$ and 66 in western NA species, as well as the Hawaiian mints. Only few chromosome numbers have been reported from Meso-South American (Meso-SA) *Stachys* species and they vary from $2n = 32$ to $2n = 80–82$ or 84 (Mulligan and Munro, 1989; Lindqvist and Albert, 2002).

The Hawaiian endemic mints consist of 59 species in three genera, *Haplostachys*, *Phyllostegia* and *Stenogyne*, characterized by a high degree of morphological variation contrasted with a low level of genetic diversity (Lindqvist and Albert, 2002; Lindqvist et al., 2003, 2006, 2007). Our previous studies have shown that the Hawaiian mints are monophyletic, having colonized the Hawaiian Islands once during the early to late Pliocene, and possibly having originated from one or more hybridization events involving temperate NA and Meso-SA *Stachys* members (Lindqvist and Albert, 2002; Lindqvist et al., 2003, 2006, 2007; Roy et al., 2013). Based on cpDNA and nrDNA sequences, we showed that there were at least two independent migration events of *Stachys* into the NW, once during middle to late Miocene and another toward the beginning of Pliocene (Lindqvist and Albert, 2002; Roy et al., 2013). Descendants of the first migration survived only in Meso- and South America (SA), whereas the latter event colonized different parts of temperate North America.

In this study, we have further disentangled the phylogenetic relationships among the NW *Stachys* and the Hawaiian endemic mints through low-copy nuclear genes. Our main goals were: (1) To reassess the origin and ancestry of the Hawaiian mints, tracing their evolutionary relationships within their closest NW *Stachys* relatives; (2) Investigate whether hybridization or incomplete lineage sorting (ILS) leads to the phylogenetic incongruence of these groups of lamioid mints when markers from different genomic regions are used; and (3) Delineate further the reticulate evolutionary relationships observed between the Mesoamerican and SA *Stachys*. We utilized five low-copy nuclear loci: (1) *AFO* (putative homolog of the axial regulator ABNORMAL FLORAL ORGANS; also known as FILAMENTOUS FLOWER or YABBY1), spanning the last intron and exon, (2) a region of *WAXY* (also known as Granule Bound Starch Synthase or GBSSI), spanning exons 11–13, (3) the third intron of *NIA* (Nitrate reductase), (4) intron regions of *COR* (Cold acclimation protein), and (5) intron regions of *ADK* (Adenosine kinase). *NIA* in most land plants have been previously shown to act as a homodimer utilizing NADH as a cofactor, to catalyze the first reaction in the uptake of nitrogen from the soil, the reduction of nitrate to nitrite (Hoff et al., 1992). A previous study by Howarth and Baum (2005) concluded that the third intron of *NIA* (*NIA*-i3) was the most divergent marker for phylogenetic analyses among the three *NIA* introns tested in *Scaevola* (Goodeniaceae). Although *NIA* appears to be single-copy in the majority of genera studied, there may be duplicate copies present in plants with polyploid origins (Levin et al., 2009). The *WAXY* locus (GBSSI) has also been used widely in phylogenetic reconstructions in a variety of plant groups, and has been shown to exist either as single or duplicate copies (Mason-Gamer et al., 1998; Mason-Gamer, 2013; Yuan and Olmstead, 2008; Levin et al., 2009). The other two low-copy loci *ADK* and *COR* were used by Curto and Colleagues (2012) in a phylogenetic study of subfamily

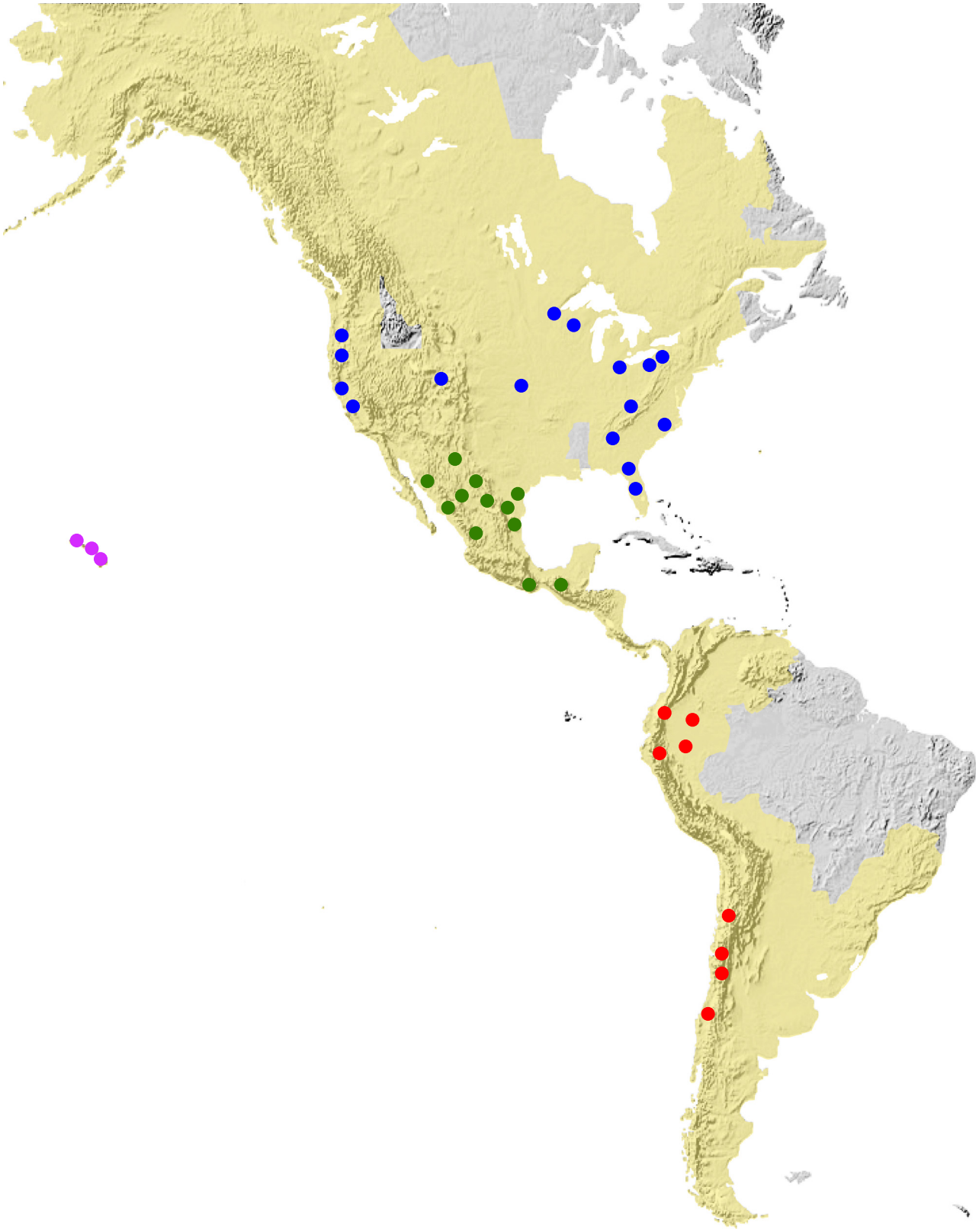

Fig. 1. Map of the geographic distribution of the NW and Hawaiian Stachydeae members, as well as those sampled in our study. The yellow shaded area represents their overall geographic distribution, whereas the circles represent the collecting localities. Blue represents those sampled from temperate NA; Green represents those from Mesoamerica; Red represents those from SA; Purple represents those sampled from Hawaii.

Nepetoideae (Lamiaceae). These two loci consist of intron regions, flanked by exons that provide conserved primer binding sites (EPIC markers; Curto et al., 2012; Thomson et al., 2008). Both these markers have been suggested as potentially phylogenetically informative providing a substantial amount of variation among closely related species. The *AFO* locus, in particular its 3′ UTR region, has been observed to contain extensive length and sequence variation by Lindqvist and Colleagues (2006), who

proposed its potential utility in phylogenetic reconstructions among closely related species, and our current research is one of the first instances of successful utilization of this locus for phylogenetic assessment.

2. Materials and methods

2.1. Taxon sampling, DNA extraction, amplification, and sequencing

All taxon names in this present study follow the "World checklist of Lamiaceae and Verbenaceae" (Govaerts et al., 2013). Sequence data were collected for a total of 66 accessions, including 65 species for *ADK* (representing 17 Hawaiian species, 12 OW species, 14 NA species and 22 Meso-SA), 63 species for *AFO* (17 Hawaiian species, 12 OW species, 14 NA species and 20 Meso-SA), 64 species for *NIA* (17 Hawaiian species, 12 OW species, 14 NA species and 21 Meso-SA species), 66 species for *COR* (18 Hawaiian species, 12 OW species, 14 NA species and 22 Meso-SA species), and 61 species for *WAXY* (17 Hawaiian species, 11 OW species, 13 NA species and 20 Meso-SA) (Fig. 1). Leaf material was collected from specimens held at the following herbaria: A, AAU, BISH, C, LL, M, NY, RM, TEX, US, UPS, UNA, and UTC (abbreviations followed from Holmgren et al., 1990) (Table 1). In certain cases, fresh leaf material further dried in silica gel was obtained from Hawaiian mints collected during fieldwork (with permissions granted by the Department of Land and Natural Resources, State of Hawaii, P.O. Box 4849, Hilo, Hawaii 96720 USA) and of cultivated *Stachys*. The sampling for the five different datasets was different for a few taxa due to limitations in the availability of material. However, based on previous studies (Lindqvist and Albert, 2002; Roy et al., 2013) and topological congruence in the overall placement of taxa within their respective clades, we expect that placement of these few missing taxa will follow other close relatives included in the analyses. In preliminary analyses also including the monotypic genus *Melittis melissophyllum*, which has been shown by Scheen et al. (2010) to be sister to the rest of the Stachydeae lineage (not shown), the highly divergent OW species *Stachys inflata* and *S. lavandulifolia* constituted a sister lineage to all other included taxa, and were used to root the phylogenetic trees in this study. DNA was extracted from silica-dried leaves or from herbarium specimen leaf fragments using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The primers used in the amplification of the five loci have been published previously (Table 2), except in the case of *NIA*. After initial amplification of *NIA* using the primers from Howarth and Baum (2002), a reverse primer was specifically designed for our study (Table 2). All loci were amplified using a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). A touchdown method was used with the following thermocycling profile: hold for 10 min at 95 °C; 10 cycles of 1 min at 95 °C, 1 min at 60 °C and decreasing the temperature by 1 °C every cycle, 1 min at 72 °C; followed by 35 cycles of 1 min at 94 °C, 1 min at 50 °C, 1 min at 72 °C; extension of 1 min at 72 °C. In certain cases when this touchdown method failed to amplify our loci of interest, a modified touchdown method was used, where the annealing temperature started at 55 °C decreasing by 1 °C every cycle. PCR products were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

All PCR products generated were further cloned using the Qiagen PCR cloning kit (Qiagen, Hilden, Germany) following the manufacturer's instructions, except for using 25 μl competent cells to transform each ligation reaction. Transformed clones were incubated overnight at 37 °C. Up to 12 positive clones were picked per species, with the average number of clones varying between 2 and 4 per locus (Supplementary Table S1). PCR reactions were prepared in 25 μl volumes with the AmpliTaq DNA Polymerase buffer II kit (Applied Biosystems, Foster City, CA, USA) using 2.5 μl buffer, 2.5 μl $MgCl_2$, 1.0 μl dNTP, 0.6 μl each of M13F and M13R primers and 0.2 μl of AmpliTaq polymerase. All PCR products were examined by gel electrophoresis on 1% agarose gels and positive PCR amplified products were sequenced in one direction using SP6 or T7 primers at the University of Washington High Throughput Genomics Center at Seattle, USA.

2.2. Phylogenetic gene tree reconstruction

All sequences generated were edited and assembled in the program Sequencher v.4.7 (Gene Codes, Ann Arbor, Michigan, USA), aligned with ClustalX v.2 (Larkin et al., 2007) or MAFFT (EMBL-EBI), and alignments were manually adjusted in BioEdit (Hall, 1999). Gaps were treated as missing and indels were not coded. Except for a few cases, all the cloned sequences from individual accessions formed their own clades (not shown), and a single representative clone was picked randomly for each group of clones, and the final datasets used for all the phylogenetic analyses consist of these single representative clones. Significant incongruence was observed among all five datasets/tree topologies, observed from one-tailed Shimodaira–Hasegawa (SH) tests (Shimodaira and Hasegawa, 1999) implemented in PAUP* v.4.0b10 (Swofford, 2002) (all $P < 0.001$). Hence, the data from the five loci were not concatenated to run further analyses. If detected, genetic recombination, which suggests a departure from neutrality, may lead to alternative explanations for incongruence in phylogenetic datasets. We evaluated evidence of recombination using the Phi test (Bruen et al., 2006) in Splitstree v.4.13.1 (Huson, 1998).

Phylogenetic relationships were examined for the five loci separately using Bayesian inference conducted in MrBayes v.3.1.2 (Huelsenbeck and Ronquist, 2001). We used substitution models that best fit the data as determined by the Bayesian Information Criterion (BIC) using the program jModeltest v.1.1 (Posada, 2008). The TPM1uf + G model was favored for the *AFO* dataset, HKY + G for *ADK* dataset, TIM + G for *NIA*, TIM3 + G for *WAXY* and TPM1uf + G for *COR* dataset. However, since the TPM1uf + G, TIM + G, TIM3 + G models are not implemented in MrBayes v.3.1.2 (Huelsenbeck and Ronquist, 2001), we used the models with the next highest BIC score, which were GTR + G for the first three loci (i.e., *AFO*, *NIA* and *WAXY*, respectively) and HKY + G for *COR*. The Bayesian analyses were run with two Markov Chain Monte Carlo (MCMC) runs for ten million generations each. Trees were sampled every 500 generations, the program Tracer v.1.5 (Drummond and Rambaut, 2007) was used to check for stationarity, and the burn-in value for obtaining a 50% majority rule consensus tree was set to ignore the first 25% of trees to include trees only after stationarity was reached. Clade support was determined by Bayesian posterior probabilities (PP; Rannala and Yang, 1996). The 50% majority rule consensus trees were viewed with FigTree v.1.4.0 (Rambaut, 2008).

2.3. Divergence time estimation

Estimation of divergence times was obtained using the program BEAST v.1.8.0 (Drummond and Rambaut, 2007). This method simultaneously estimates phylogeny and molecular rates using a Markov Chain Monte Carlo strategy. The analyses were run on each of the *WAXY*, *ADK* and *COR* datasets, for which we also had sequences from *Melittis melissophyllum*. To estimate divergence times, a Yule process speciation prior and an uncorrelated lognormal (UCLN) model of rate change with a relaxed clock (Drummond et al., 2006) was used and the analyses were run for 30 million generations with parameters sampled every 1000 generations. Trace

Table 1
List of taxa, voucher information and GenBank accession numbers.

Taxa list	Voucher information	Geographic distribution/ Collecting locality	Genbank accession numbers				
			WAXY	*ADK*	*COR*	*AFO*	*NIA*
Haplostachys haplostachya (A. Gray) St. John	V. A. Albert, M. Bendiksby, C. Lindqvist, A. C. Scheen *HI03-33 (NTBG #950403)	Hawaii/Hawai'i	KP063817	KM978834	KP063672	KP063589	KP063748
Melittis melissophyllum L.	S. Vautier & J. P. Bersier 2896805 (US)	W. & S.W. Europe to Turkey/France	KP063884	KP063882	KP063883		
Phyllostegia ambigua (A. Gray) Hillebr.	V. A. Albert, M. Bendiksby, C. Lindqvist, A. C. Scheen *HI03-59 (Volcano Research Station #000606)	Hawaii/Hawai'i	KP063818	KM978835	KP063673	KP063590 KP063591	KP063749
Phyllostegia electra C. Forbes	K. Wood 2967 (BISH)	Hawaii/Kaua'i	KP063819	KM978836 KM978837	KP063674 KP063675	KP063592 KP063593	KP063750
Phyllostegia kaalaensis St. John	S. Perlman 6117 (BISH)	Hawaii/O'ahu	KP063820	KM978838 KM978839	KP063676	KP063594 KP063595	KP063751
Phyllostegia racemosa Benth.	V. A. Albert, M. Bendiksby, C. Lindqvist, A. C. Scheen *HI03-57 (Volcano Research Station #95060)	Hawaii/Hawai'i,	KP063821	KM978840	KP063677 KP063678	KP063596 KP063597	KP063752
Phyllostegia velutina (Sherff) St. John	V. A. Albert, M. Bendiksby, C. Lindqvist, A. C. Scheen *HI03-61 (Volcano Research Station #960302)	Hawaii/Hawai'i	KP063822	KM978841	KP063679	KP063598	KP063753
Phyllostegia waimeae Wawra.	V. A. Albert, M. Bendiksby, C. Lindqvist, A. C. Scheen *H103-27 (NTBG #010580)	Hawaii/Kaua'i	KP063823	KM978843 KM978843	KP063680	KP063599 KP063600	KP063754
Stachys aculeolata Hook f.	Y. B. Harvey, G. Mungai & K. Vollesen (C)	E.C. to E. tropical Africa/ Kenya Naivasha Distr.	KP063824	KM978844	KP063681	KP063601	KP063755
Stachys aethiopica L.	Børge Petterson 2146 (UPS)	S. Tropical to S. Africa/ Mocambique	KP063825	KM978845	KP063682	KP063602	KP063756
Stachys affinis Bunge	C. Lindqvist and V. A. Albert 359 (UNA), from Companion Plants	China to N. Myanmar/ Cultivated unknown source (East Asia)	KP063826	KM978846	KP063683	KP063603	KP063757
Stachys agraria Schltdl. & Cham	B. Ertter 5638 (UTC)	Texas to Guatemala/Texas Nueces Co.	KP063827	KM978860	KP063697	KP063617	KP063769
Stachys ajugoides var. *rigida* Jeps. & Hoover	C. Lindqvist 11-02 (UB)	W. USA to Mexico (Baja California)/Cultivated unknown source	KP063828	KM978847	KP063684	KP063604	KP063758
Stachys albens A. Gray	C. Lindqvist 11-06 (UB)	California/Cultivated unknown source	KP063830	KM978847	KP063685	KP063605	KP063759
Stachys albotomentosa Ramamoorthy	H. Rubio 1984 (TEX)	N.E. Mexico/Mexico, Landa Mun.	KP063829	KM978849 KM978850	KP063686	KP063606	KP063760
Stachys alpigena T.C.E.Fr.	Olof Ryding 2133 (UPS)	Ethiopia to Rwanda/ Ethiopia	KP063831	KM978851	KP063687	KP063607	KP063761
Stachys arvensis (L.) L.	Olof Ryding 2394 (C)	Macaronesia to Taiwan/ Canary Islands, Tenerife	KP063832	KM978852	KP063688	KP063608	KP063762
Stachys aspera Michx.	J. B. Nelson 1326 (UNA)	N.C to E. USA/Florida, Leon Co.	KP063833	KM978853	KP063689	KP063609	KP063763
Stachys biflora Hook. & Arn.	G. B. Hinton et al. 24561 (TEX)	Mexico/Mexico, Nuevo Leon		KM978868	KP063704		KP063776
Stachys bigelovii A. Gray	G. B. Hinton et al. 19781 (TEX)	S. Texas to N. Mexico/ Mexico, Nuevo Leon	KP063834	KM978855	KP063691	KP063611	KP063765
Stachys bullata Benth.	M. R. Crosby & N. Morin 14355 (RM)	W. California/California, Monterey Co.	KP063835			KP063652	
	C. Lindqvist 11-02 (UB)	W. California/California, Monterey Co.		KM978856	KP063692		KP063766
Stachys chamissonis Benth.	C. Lindqvist 10-02 (UB)	W. Canada to W. USA/ Cultivated (from R. Olmstead)	KP063836	KM978857	KP063693 KP063694	KP063612 KP063613	KP063767
Stachys coccinea Ort.	C. Lindqvist and V. A. Albert *355 (New York Botanical Garden #911/97A)	Arizona to Texas and C. America/Cultivated unknown source	KP063837	KM978858 KM978859	KP063695 KP063696	KP063614 KP063615	KP063768
Stachys cordata Riddell	J. B. Nelson 14361 (UNA)	N.C. and E. USA/South Carolina, Richland Co.	KP063838	KM978882	KP063718	KP063642	KP063790 KP063791
	A. L. Moldenke & H. N. Moldenke 27394 (AAU)	N.C. and E. USA/USA, Ohio, Coshocton Co.	KP063839	KM978886	KP063722	KP063642	KP063794
Stachys debilis Kunth.	C & E. Franquemont 106 (AAU)	Equador/Ecuador, Chulkunag, Punin, Chimborazo	KP063840	KM978861	KP063698	KP063618	KP063770
Stachys drummondii Benth.	B. Ertter 5530 (UTC)	Texas to N.E. Mexico/Texas, Cameron Co.	KP063841	KM978862 KM978863	KP063699	KP063619	KP063771
Stachys elliptica Kunth.	B. Ollgaard et al. 91045 (AAU)	Equador/Ecuador, Prov Loja	KP063842 KP063843	KM978864	KP063700	KP063620	KP063772
Stachys eriantha Benth.	A. McDonald & G. Nesom 2495 (TEX)	Mexico, Colombia to NW. Venezuela and Ecuador/ Mexico, Chihuahua	KP063844	KM978865	KP063701	KP063621	KP063773
Stachys floridana Shuttlw. Ex. Benth.	M. Kortright 102 (UNA)	Florida/Alabama, Tuscaloosa Co.	KP063845	KM978866	KP063702	KP063622	KP063774

Table 1 (*continued*)

Taxa list	Voucher information	Geographic distribution/ Collecting locality	Genbank accession numbers				
			WAXY	*ADK*	*COR*	*AFO*	*NIA*
Stachys gilliesii Benth.	S. Pfanzelt 241 (M)	W. South America to Chile and Brazil/Region Metropolitana, Chile	KP063846	KM978867	KP063703	KP063623	KP063775
Stachys grandidentata Lindl.	Walter J. Eyerdam 10081 (US)	N. and C. Chile/Chile	KP063847	KM978869	KP063705	KP063624	KP063777
Stachys hamata Epling	B. Lojtnant et al. 11835 (AAU)	Colombia to Ecuador, NE. Venezuela/Ecuador, Prov Carchi, La Esperanza	KP063848	KM978870	KP063706	KP063625	KP063778
Stachys hispida Pursh.	S. R. Zeigler & M.F.Leykern 1963 (AAU)	C. & E. Canada, N.C. & E. USA/Wisconsin, La Crosse	KP063849	KM978871	KP063707	KP063626	KP063779
Stachys inflata Benth.	J.S. Anderson & I.C. Petersen 68 (AAU)	N.E. Turkey to Iran/Iran	KP063850	KM978872	KP063708	KP063627	KP788034
Stachys kouyangensis (Vaniot) Dunn	B. Bartholomew et al. Sino Amer. Bot. Exp. 1362 (US)	Tibet, SC. China to Indo-China/China, Yunnan	KP063851	KM978873	KP063709	KP063628	KP063781
Stachys lamioides Benth.	L. Holm-Nielsen & J. Jaramillo 23323 (AAU)	Colombia to Ecuador/ Equador, Prov Imbabura	KP063852	KM978874	KP063710 KP063711	KP063629	KP063782
Stachys langmaniae Rzed. and Calderon	McDonald 1620 (TEX)	N.E. Mexico/Mexico, Nuevo Leon	KP063853	KM978875	KP063712	KP063630	KP063783
Stachys latidens Small	J. A. Churchill 83034 (RM)	N.C. and E. USA/Carolina, Mitchell Co.	KP063854	KM978876	KP063713	KP063631	KP063784
Stachys lavandulifolia Vahl.	J. S. Andersen & I. C. Petersen 31 (AAU)	S. & E. Turkey to Iran/Iran, Semnan	KP063855	KM978877	KP063714	KP063632	KP063785
Stachys lindenii Benth.	R. Torres C. 4602 (TEX)	S. Mexico to Guatemala/ Mexico, Oaxaca				KP063633	
	P. Tenorio L. 11084 (TEX)	S. Mexico to Guatemala/ Mexico, Oaxaca	KP063856 KP063857	KM978878 KM978879	KP063715		KP063786
Stachys macraei Benth.	M. Mahn 3925 (AAU)	C. & S. Chile/Chile, Santiago	KP063858	KM978880	KP063716	KP063634	KP063787
Stachys mexicana Benth. (cf. *chamissonis*)	R. E. Brooks 20248 (RM)	Alaska to California/Oregon, USA	KP063859 KP063860	KM978881	KP063717	KP063635 KP063636	KP063788 KP063789
Stachys pacifica B. L. Turner	G. Flores F. 2344 (TEX)	W. Mexico/Mexico, Nayarit	KP063861	KM978883	KP063719	KP063638 KP063639	KP063792
Stachys pilosa Nutt.	G. A. Wheeler 11047 (AAU)	Alaska to USA/Minnesota, Traverse Co.	KP063862	KM978884	KP063720	KP063640	KP063793
Stachys radicans Epling	D. E. Breedlove 51924 (TEX)	Mexico, Colombia/Mexico, Chiapas	KP063863	KM978885	KP063721	KP063641	
Stachys rigida subsp. *rigida*	A. Tiehm 12609 (UTC)	Washington to N. California/Sierra Nevada, Washoe Co	KP063865	KM978887	KP063723	KP063643	KP063795
Stachys riederii var. *riederii*	H. Takahashi 2950 (C)	Siberia to Japan/Japan, Hokkaido	KP063864	KM978854	KP063690	KP063610	KP063764
Stachys rothrockii A. Gray	E. Neese et al. 15716 (TEX)	Utah to New Mexico/Utah, Cane Co.	KP063866	KM978889	KP063725	KP063645	KP063797
Stachys rotundifolia Moc & Sesse ex Benth.	D. E. Breedlove 55575 (TEX)	C. & S. Mexico/Mexico, Chiapas	KP063867	KM978888	KP063724	KP063644	KP063796
Stachys sericea Cav.	F. C. Joseph 4327 (US)	C. Chile/Chile		KM978890	KP063726		KP063799
Stachys strictiflora C. Y. Wu	Charlotte Lindqvist 10-07 (UB)	China, Yunnan/Cultivated unknown source	KP063868	KM978891	KP063727	KP063646	KP063800
Stachys sylvatica L.	C. Lindqvist & V. A. Albert 358 (UNA)	Macaronesia, Europe to W. Himalaya/Cultivated unknown source	KP063869	KM978892	KP063728	KP063647	KP063801
Stachys tenuifolia Willd.	John. B. Nelson (UNA)	E. Canada, C. & E. USA/ Florida	KP063870	KM978893	KP063729		KP063802
	R. L. Mc.Gregor 31847 (RM)	E. Canada, C. & E. USA/ Kansas, Montgomery Co.				KP063648	
Stachys torresii B. L. Turner	A. McDonald 2927 (TEX)	Mexico/Mexico, Oaxaca	KP063871	KM978894	KP063730	KP063649 KP063650	KP063803
Stachys vulnerabilis Rzed. & Calderon	G. B. Hinton et al. 24774 (TEX)	N.E. Mexico/Mexico, Nueva Leon	KP063872	KM978895 KM978896	KP063731	KP063651	KP063804
Stenogyne bifida Hillbri.	K. Wood 6284 (BISH)	Hawaii/Moloka'i	KP063873			KP063653 KP063654	
	V. A. Albert, M. Bendiksby, C. Lindqvist, A. C. Scheen *HI03-32 (NTBG #8-7-98)	Hawaii/Moloka'i		KM978897	KP063732		KP063805 KP063806
Stenogyne calaminthoides A. Gray	C. Lindqvist & V. A. Albert 82 (NY)	Hawaii/Hawai'i				KP063655 KP063656	
	V. A. Albert, M. Bendiksby, C. Lindqvist, A. C. Scheen *HI03-04	Hawaii/Hawai'i	KP063874	KM978898	KP063733 KP063734		KP063807
Stenogyne campanulata Weller & Sakai	V. A. Albert, M. Bendiksby, C. Lindqvist, A. C. Scheen *HI03-36 (NTBG #030081)	Hawaii/Kaua'i	KP063875	KM978899	KP063735 KP063736	KP063657 KP063658	KP063808
Stenogyne kamehamehae Wawra	S. Perlman 6933 (BISH)	Hawaii/Maui			KP063737 KP063738		
Stenogyne kanehoana Degener & Sherff	J. Obata 356 (BISH)	Hawaii/O'ahu	KP063876	KM978900 KM978901	KP063739	KP063659 KP063660	KP063809
Stenogyne microphylla Benth.	C. Lindqvist & V. A. Albert 85 (NY)	Hawaii/Hawai'i	KP063877	KM978902	KP063740	KP063661 KP063662	KP063810

(*continued on next page*)

Table 1 (*continued*)

Taxa list	Voucher information	Geographic distribution/ Collecting locality	Genbank accession numbers				
			WAXY	*ADK*	*COR*	*AFO*	*NIA*
Stenogyne purpurea H. Mann	K. Wood 1772 (BISH)	Hawaii/Kaua'i				KP063663 KP063664	
	V. A. Albert, M. Bendiksby, C. Lindqvist, A. C. Scheen *HI03-40	Hawaii/Kaua'i	KP063878	KM978903 KM978904	KP063741 KP063742		KP063811
Stenogyne rugosa Benth.	V. A. Albert, M. Bendiksby, C. Lindqvist, A. C. Scheen *HI03-65 (Volcano Rare Plant Facility #021207)	Hawaii/Hawai'i			KP063743 KP063744	KP063665 KP063666	KP063812
	V. A. Albert, M. Bendiksby, C. Lindqvist, A. C. Scheen *HI03-73	Hawaii/Hawai'i	KP063879	KM978905			
Stenogyne scrophularioides Benth.	V. A. Albert, M. Bendiksby, C. Lindqvist, A. C. Scheen *HI03-58 (Volcano Rare Plant Facility #020423)	Hawaii/Hawai'i	KP063880	KM978906	KP063745	KP063667 KP063668	KP063813
Stenogyne sessilis Benth.	V. A. Albert, M. Bendiksby, C. Lindqvist, A. C. Scheen *HI03-67 (Volcano Rare Plant Facility #021212)	Hawaii/Hawai'i	KP063881	KM978907	KP063746	KP063669 KP063670	KP063814 KP063815
Suzukia shikikunensis Kudo	Chii-Cheng Liao et al. 564 (A)	C. & E. Taiwan/Pingtung Hsien, Taiwan	KP063882	KM978908	KP063747	KP063671	KP063816

* = Designates the field collection number, NTBG = National Tropical Botanical Garden.

Table 2
List of primer sequences used in this study.

Locus name	Forward primer sequence (5′-3′)	Reverse primer sequence (5′-3′)	Reference and original name used
NIA	AARTAYTGGTGYTGGTGYTTYTGGTC	TGCACAAYGTCAATTATTTTATTCAAG	Howarth and Baum (2002) (forward primer); this study (reverse primer)
AFO	CATAGTAACAGATGAACCCTAA	ATCAACCCGTGAAGAAACC	Lindqvist et al. (2006)
WAXY	TAYACTGGSTTCCAYATGG	GGAGTGGCRACGTTTTCCTT	Tank and Olmstead (2009); *GBSSI*
COR	CTCGAATGTGTTCCTGCAG	CACATCCCTCTTAGTCCCATAC	Curto et al. (2012); S.milt 001
ADK	GCTCCATTTATCTGTGAGTTC	CAACTGTGATAAAAATCCACC	Curto et al. (2012); M.pip. 047

files were loaded into Tracer v.1.5 (Drummond and Rambaut, 2007) to look for an Effective Sampling Size (ESS) greater than 200 for all parameters sampled from the MCMC, and to examine the posterior distributions of all parameters and their associated statistics including 95% highest posterior density (HPD) intervals. To optimize efficiency in BEAST, we initially undertook several trial runs of 10–20 million generations and analyzed the results using the program Tracer v.1.5 (Drummond and Rambaut, 2007). These results were then used to determine the number of generations necessary to achieve the desired effective sampling size (ESS) of at least 200 and to optimize the operator settings for our above mentioned final analysis. The program TreeAnnotator v.1.8.0 (Drummond and Rambaut, 2007) was used to summarize the set of post burn-in trees and their parameters (burn-in set to 3000), to produce a maximum clade credibility (MCC) chronogram showing mean divergence time estimates with 95% HPD intervals. The program FigTree v.1.4.0 (Rambaut, 2008) was used for visualization of the resulting divergence timings. Lamiales have been elusive in terms of reliable fossil records, and there is scant existence of some old and reliable fossils (Mai, 2001). The oldest reliable fossil has been sampled from the Seravallian Age of the Middle Miocene flora of Germany and belongs to *Stachys laticarpa* (seed/fruit) and *Lamium* sp. (13.8-11.6 Million years ago (Mya); Mai, 2001). We used the *Stachys laticarpa* fossil as a calibration point (13.8 Mya) to constrain the crown group of the *Stachys* s.l. clade (Stachydeae excluding *Melittis*). To reflect the uncertainty related to the fossil data, we set lognormally distributed priors for our calibration with the following value for the offset O = 13.8, with Standard deviation = 0.8 and Mean = 0.5.

2.4. Species tree and network reconstruction

We also implemented a multi-locus coalescent model to estimate a "species tree" and to further explore phylogenetic signals in our data. For this purpose we only included species for which all five loci were recovered. We used the program *BEAST (Heled and Drummond, 2010), within the BEAST v.1.8.0 software package (Drummond and Rambaut, 2007), to estimate a species tree from five separate loci, that were treated as unlinked. *BEAST applies Bayesian MCMC analysis of the sequence data, jointly exploring gene trees and species trees to estimate the species tree posterior distribution under the assumption of the coalescent model. A relaxed molecular clock model for all loci and the GTR model of nucleotide substitution were applied. The tree prior was set to exponential, and other priors were kept to default values. Analyses were done for 50 million generations sampling every 10,000 generations. A relative proportion of the posterior samples from each Markov chain were discarded as burn-in, and trees were summarized in TreeAnnotator v.1.8.0 (Drummond and Rambaut, 2007). The resulting species tree was then visualized in FigTree v.1.4.0 (Rambaut, 2008). We also utilized the Species TRee Analysis Web server (STRAW) (Shaw et al., 2013) as a second method to create a species tree from our individual gene trees. Within STRAW, we utilized the STAR (Liu et al., 2009) method for species tree reconstruction, which uses average ranks of gene coalescence times to build species trees from a set of rooted gene trees. For this purpose we used rooted maximum likelihood trees from our individual pruned datasets (refer to Section 2.2) for our five loci, built through the RAxML Blackbox web server (Stamatakis et al., 2008).

Phylogenetic models usually assume a hierarchical, bifurcating tree that may not apply to our lineages in question. Reticulate networks aid in providing an in-depth picture of evolutionary history, in which the internal nodes within the network represent ancestral species, and nodes with more than two parents correspond to reticulate events such as hybridization. Hence, we explored a phylogenetic network method to analyze signals of reticulate evolution and character conflicts in our datasets. The network was created with Neighbor-Net (Huson and Bryant, 2006) in

SplitsTree v.4.13.1 (Huson, 1998) using uncorrected *p*-distances. We used a concatenated dataset with all the five loci for this analysis. For this analysis we discarded the highly divergent OW taxa. For distance calculations we chose the most parameterized model available in SplitsTree with an HKY85 model, transitions: transversions weighted 2:1, gamma model of rate heterogeneity, and base frequencies estimated empirically.

3. Results

3.1. Phylogenetic analysis

The total aligned positions including gaps for *AFO*, *ADK*, *NIA*, *COR*, and *WAXY* matrices were composed of 351, 763, 671, 775, and 537 characters, respectively, totaling 3.1 Kb. We observed similar overall topologies from the 50% majority rule consensus trees obtained from the Bayesian MCMC analyses of four out of the five loci (Fig. 2A–D). In each of the trees obtained from *AFO*, *ADK*, *COR* and *WAXY*, two clades of Meso-SA *Stachys* taxa (Meso-SA I and II, Fig. 2A–D respectively) (accessions with distributions ranging from Southwestern United States to Mexico/Central America, and South America) emerge, along with a single clade of well supported temperate North American (NA) *Stachys* species. This biogeographic pattern corroborates results from previous studies based on nrDNA data (Lindqvist and Albert, 2002; Lindqvist et al., 2003; Roy et al., 2013). On the contrary, the *NIA* Bayesian tree (Fig. 2E) shows two distinct clades of temperate NA mints and a single clade of Meso-SA taxa. SH tests suggested the individual gene trees to be highly incongruent to each other (all $P < 0.001$). This conflict is particularly reflected in the positions of the Hawaiian taxa with respect to the NW *Stachys* species. Unlike our previous studies (Lindqvist and Albert, 2002; Lindqvist et al., 2003; Roy et al., 2013), which showed the Hawaiian taxa to be monophyletic and nested within either the temperate NA *Stachys* (nrDNA trees) or grouped with the Meso-SA mints (cpDNA trees), the individual gene trees from the Bayesian MCMC analyses in our current study show the Hawaiian species to be polyphyletic and interspersed within both the temperate NA and the Meso-SA clades (Fig. 2A–E; Table S2). Within the major clades, relationships still remain largely unresolved and several taxa exhibit inconsistent positions among the different gene trees, although the overall biogeographic patterns are maintained. Our datasets do not show any significant instances of recombination.

3.1.1. Gene trees and the placement of the OW taxa

The tropical African species *S. aculeolata*, *S. alpigena* and *S. aethiopica* comprise a strongly supported group but with different placements in the individual gene trees. In the *WAXY* and *ADK* trees it is sister to or nested within a clade of Meso-SA II *Stachys* (Fig. 2A and B, respectively), whereas it is sister to or groups outside all or most of the NW species in the *COR* and *AFO* trees (Fig. 2C and D, respectively), and nested within the NW species sister to a clade of NA and Hawaiian species in the *NIA* tree (Fig. 2E). The placements of the OW taxa *S. arvensis* and *S. sylvatica*, also vary considerably, although they usually place in the vicinity of one another outside or as sister to the NW *Stachys* clade (Fig. 2A–D), except for the *NIA* tree where *S. arvensis* emerges as sister to a large clade of most of the NW, East (E) Asian and the tropical African species, whereas *S. sylvatica* is nested inside this clade sister to the tropical African species (Fig. 2E). The E Asian *Stachys* (*S. kouyangensis*, *S. strictiflora*, *S. reideri* var. *reideri* and *S. affinis*) as well as *Suzukia* also show incongruence in their placement among the different trees, although usually grouping with temperate NA (and Hawaiian) species (*WAXY*, *ADK*, *AFO* and *NIA*; Fig. 2A, B, D and E respectively). However, in the *COR* tree (Fig. 2C), they remain sister to a large clade of temperate NA, Meso-SA I, and most of the Hawaiian species.

3.1.2. Gene trees and the placement of the NW taxa

Despite certain topological incongruences, the taxa composition within the Meso-SA clades shows an overall similarity among four of our five gene regions (*WAXY*, *ADK*, *COR* and *AFO*) (Fig. 2A–D; Table S3). The South American taxa are not monophyletic, but remain interspersed within the Mesoamerican *Stachys* species. Two distinct, well supported clades of Meso-SA taxa (Meso-SA I and II) are observed in *WAXY*, *ADK* and *COR* (Fig. 2A–C). However, in *AFO* (Fig. 2D) only one well supported Meso-SA II clade appears, whereas taxa loosely resembling those within the Meso-SA I clade in other gene trees remain in a largely unresolved polytomy, along with Hawaiian mints and NA *S. chamissonis* and *S. mexicana*. Although, the taxon compositions for the two Meso-SA clades vary among the different genes, some commonalities are observed. For example, the SA taxa *S. debilis*, *S. elliptica*, *S. eriantha*, *S. gilliesii*, and *S. macraei* consistently group in the Meso-SA I clade for all four genes (Fig. 2A–D and Table S3), and *S. hamata*, *S. grandidentata*, and *S. lamioides* group in Meso-SA II clade for *ADK*, *WAXY*, and *AFO* (Fig. 2B, A and D respectively), whereas in the *COR* tree (Fig. 2C) only *S. grandidentata* groups exclusively within the Meso-SA II clade, *S. lamiodes* groups in both Meso-SA-I and II, and *S. hamata* groups in the Meso-SA I clade, only (Table S3). Moreover, clones of *S. lindenii* emerge as polyphyletic, grouping in both Meso-SA I and II in *ADK* and *WAXY*, respectively (Fig. 2B and A). Similarly, *S. coccinea* clones group in both Meso SA-I and II in *ADK* and *COR* gene trees (2B and C). *S. drummondii* and *S. pacifica* show similar polyphyletic placements grouping with both Meso-SA I and II in *ADK* and *AFO*, respectively (Fig. 2B and D; Table S3). In the *NIA* gene tree (Fig. 2E), we observe a single, well-supported clade of Meso-SA (and Hawaiian) taxa, without further resolution. The temperate NA taxa, along with Hawaiian mints, form a well-supported clade in four of our five gene trees (Fig. 2A–D), again with the exception of *NIA*, where they form two well-supported clades (Fig. 2E). Importantly, temperate NA *S. mexicana* and *S. chamissonis* show variable positions in some of the gene trees. *S. mexicana* groups within Meso-SA I clade in the *ADK* tree (Fig. 2B), whereas clones group with temperate NA and Meso-SA II taxa in the *AFO* (Fig. 2D) gene tree. *S. chamissonis* clones also emerge polyphyletic, grouping with both temperate NA and Meso-SA I taxa in the *COR* and *AFO* gene trees (Fig. 2C and D).

3.1.3. Gene trees and the placement of the Hawaiian mint taxa

The Hawaiian mint taxa also occupy conflicting positions across the different gene trees (Fig. 2A–E; Supplementary Table S2). Unlike our previous studies (Lindqvist and Albert, 2002; Roy et al., 2013), which showed the Hawaiian taxa to be monophyletic and nested within either the temperate NA *Stachys* (nrDNA phylogenies) or grouped with the Meso-SA mints (cpDNA phylogenies), the individual gene trees from the Bayesian MCMC analyses in our current study show the Hawaiian species to be polyphyletic and placed within both temperate NA and Meso-SA clades. In four of our five gene trees (*WAXY*, *ADK*, *COR* and *AFO*; Fig. 2A–D; Table S2), Hawaiian species group with the Meso-SA I *Stachys* species, whereas in the fifth gene tree for the locus *NIA* (Fig. 2E) we observe a single polytomous clade comprised of all the Meso-SA and most of the Hawaiian taxa. However, the positions of the different Hawaiian taxa are inconsistent among all the gene trees. In the *WAXY* tree (Fig. 2A; Table S2), a well-supported clade of *Haplostachys*, *Phyllostegia* and *Stenogyne* species is nested within the Meso-SA I clade. Another set of Hawaiian taxa group in a largely unresolved clade with all the temperate NA *Stachys* and E Asian Stachydeae members. All the clones for each individual Hawaiian taxon are monophyletic in this tree (single

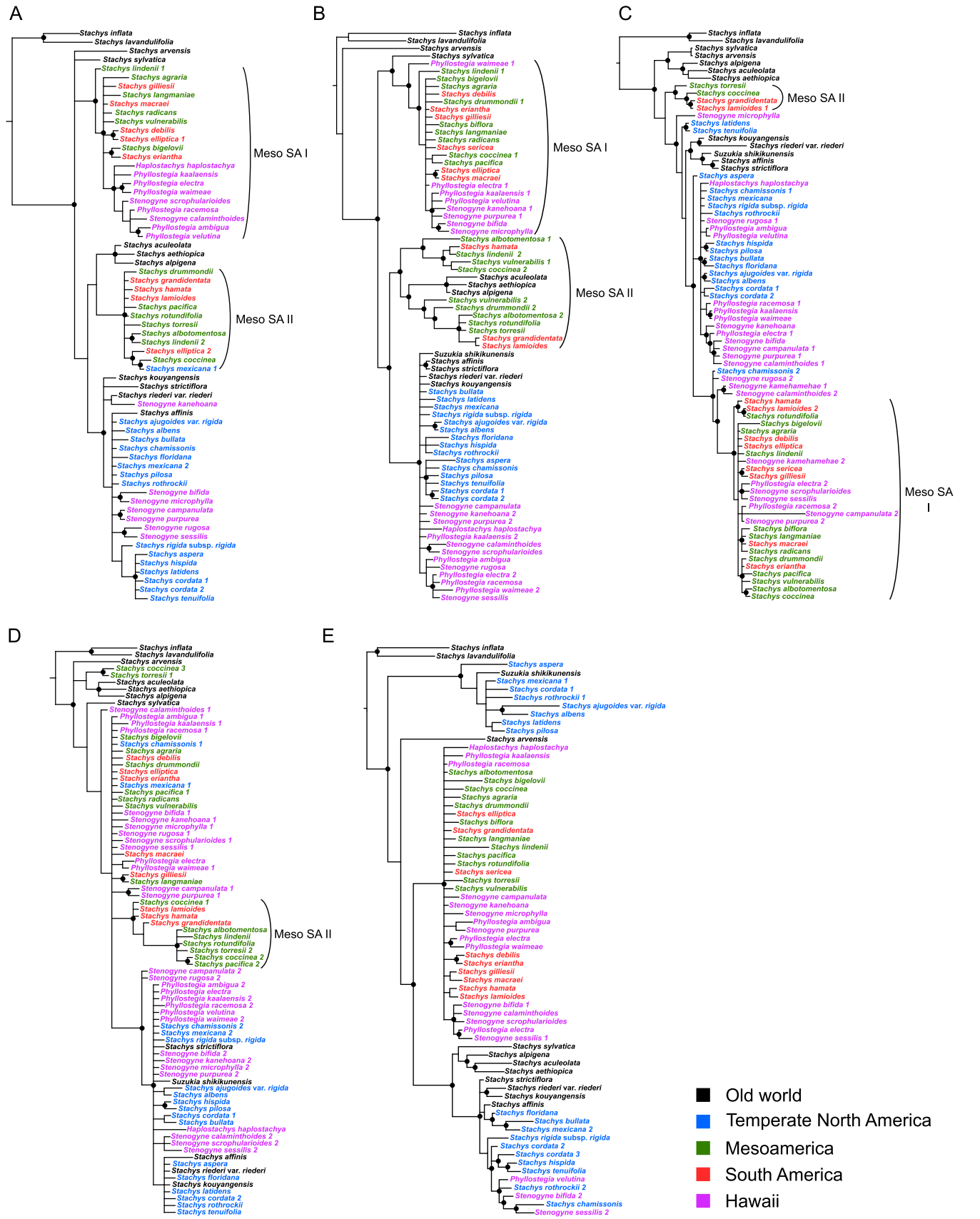

Fig. 2. (A–E) Phylogenetic trees for (A) *WAXY*, (B) *ADK*, (C) *COR*, (D) *AFO*, and (E) *NIA* loci based on the 50% majority rule consensus trees obtained from Bayesian analyses. Solid circles at nodes indicate the posterior probability values (PP) above 0.85. Numbers following taxon names refer to the different clone numbers, except for *S. cordata*, where they represent the two different accessions. Meso-SA = Meso-South America. Taxa have been color coded according to their geographic locality (see key). Meso-SA I clade also includes Hawaiian taxa in all the gene trees, and Meso-SA II clade includes OW taxa in the *ADK* tree. *S. chamissonis* and *S. mexicana* (with temperate NA distribution) are also grouped in these two clades for some of the trees.

representative clone shown in each tree; refer to Section 2.2 for details). However, in the Bayesian trees for all the other four loci (*ADK*, *COR*, *AFO* and *NIA*; (Fig. 2B–D; Table S2), individual clones of some Hawaiian taxa group both with the Meso-SA I (all Meso-SA taxa in case of *NIA*) and with the temperate NA clade. In the *ADK* tree (Fig. 2B), we observe clones of *Phyllostegia waimeae*, *P. electra*, *P. kaalaensis*, and *P. kanehoana* group closely with both the temperate NA *Stachys* and the Meso-SA I *Stachys* members. The *COR* gene tree (Fig. 2C) showed placement of clones of *Stenogyne rugosa*, *P. electra*, *P. racemosa*, *S. campanulata*, and *S. calaminthoides* with both the temperate NA and the Meso-SA I *Stachys* species. In the AFO gene tree (Fig. 2D), except *P. electra* and *Haplostachys haplostachya*, clones of all other Hawaiian taxa group with both the temperate NA and the Meso-SA I *Stachys* members. The *NIA* tree (Fig. 2E) shows *S. bifida* and *S. sessilis* clones grouping with temperate NA and Meso-SA clades of the NW *Stachys*. The other members of the Hawaiian mint genera are positioned with either the temperate NA or the Meso-SA members of *Stachys* in all the gene trees. Additional analyses excluding outlier clones of the Hawaiian taxa led to similar placements and relationships for the rest of the individuals in all the gene trees.

3.2. Species trees and network

The overall topology of our multi-locus coalescent trees (referred to as "species trees") derived from all five loci using *BEAST and STRAW (Fig. 3 and Supplementary Fig. S1) largely mirrors the nrDNA 5S-NTS Bayesian tree from our previous studies (Lindqvist and Albert, 2002; Roy et al., 2013). The species trees recovered a more lucid phylogenetic placement of the major clades with the Hawaiian mints grouping within a well-supported clade of temperate NA *Stachys*. In both species trees, no Hawaiian species group with Meso-SA clades, unlike the individual gene trees. The species trees resolved a well-supported clade comprising all temperate NA *Stachys* species, the Hawaiian mints, and the E Asian taxa (*S. strictiflora*, *S. riederi* var *riederi*, *S. kouyangensis*, and *S. affinis*). Although not well supported, the predominantly western NA *S. ajugoides* var. *rigida* and *S. albens* emerge as sister to the rest of the Hawaiian taxa along with the Hawaiian *Haplostachys haplostachya* in our *BEAST species tree. This temperate NA-Hawaiian clade is in turn sister to the two Meso-SA clades (Meso-SA I and II). Similar to some of our individual gene trees, the *BEAST and STRAW species tree places *S. debilis*, *S. eriantha*, *S. gilliesii*, *S. elliptica* and *S. macraei* within the Meso-SA I clade and *S. hamata*, *S. grandidentata* and *S. lamioides* within the Meso-SA II clade. The tropical African *S. aethopica*, *S. aculeolata* and *S. alpigena* form a well-supported clade with *S. sylvatica* and S. *arvensis,* and resolve as sister to the rest of the NW Stachydeae in our *BEAST species tree (Fig. 3).

Our Neighbor-Net network generated from a concatenated dataset (Fig. 4) summarizes the result displayed by the individual gene trees, with a distinct cluster of temperate NA (and E Asian) species, and two clusters composed of Meso-SA species. There is evidence of considerable reticulation among the Hawaiian taxa, which exhibit an intermediate placement with respect to these NW *Stachys* clusters, with connections to both the temperate NA and the Meso-SA clusters by bundles of parallel edges representing conflict regarding their origin and parental lineages.

3.3. Divergence time estimates

The divergence dating analyses based on the three loci recovered similar estimates to those observed in Roy et al. (2013) study, for two major nodes, mapped on the species tree derived from the *BEAST analysis (Fig. 3), is in congruence with our previous results with nrDNA markers (Roy et al., 2013). The individual median and 95% HPD values for each locus are listed in Table 3. Due to topological incongruence between our different datasets, we have only reported the date estimates for congruent, well-supported nodes among all loci. The estimated initial diversification of the *Stachys* crown group to the NW was between 10.9–9.9 Mya (95% HPD range between 13.2 and 6.6 Mya; Node I), which is during the late Miocene period. Further diversification into temperate North America took place between 7.1 and 3.8 Mya (95% HPD range between 9.7 to 2.3 Mya; Node II).

4. Discussion

4.1. Origin of the Hawaiian mints: Hybridization or incomplete lineage sorting (ILS)?

We observed substantial topological incongruence among the Bayesian trees derived from the five low-copy nuclear loci (Fig. 2A–E). Other examples of incongruence within Lamiales have been observed in families such as Plantaginaceae (Albach and Chase, 2004; Blanco-Pastor et al., 2012), Scrophulariaceae (Scheunert and Heubl, 2014), and Lamiaceae (Bräuchler et al., 2010). Incongruent patterns among the different loci in these studies led the authors to conclude that hybridization and polyploidization and/or incomplete lineage sorting played an active role in the speciation and diversification processes within these groups. Incongruence in our study is mainly reflected in the polyphyletic positions of the Hawaiian species, which are nested within both the temperate NA and Meso-SA I clade in the individual gene trees (Fig. 2A–E; Table S2). This pattern is unlike our previous studies, where the Hawaiian mints were monophyletic, nested within either the temperate NA *Stachys* with nrDNA data or with the Meso-SA *Stachys* in the cpDNA trees (Lindqvist and Albert, 2002; Lindqvist et al., 2003; Roy et al., 2013).

Hybridization and the resultant gene flow is often inferred to cause topological incongruence between gene trees (Slatkin and Maddison, 1989). However, conflicting phylogenetic signals among loci can also result from a variety of other processes, the most potent being incomplete lineage sorting (ILS) (Maddison, 1997; Linder and Rieseberg, 2004; Maddison and Knowles, 2006). Distinguishing between hybridization and ILS is critical when investigating species relationships at deeper levels, but is often rendered a difficult task due to the paucity of processes incorporating both (Choleva et al., 2014). In the following discussion we have made an effort to elucidate that hybridization, apart from the possibility of ILS, may have been a formidable factor influencing species relationships among the Hawaiian mints and the NW *Stachys*, leading to the observed discrepancies in our data and a complex ancestry of the Hawaiian taxa from within the NW *Stachys*.

Gene tree heterogeneity, usually resulting from incomplete sorting of ancestral alleles, is common in cases of rapid speciation, such as the one that likely gave rise to the NW *Stachys* and the Hawaiian mints (Degnan and Rosenberg, 2009). Our previous study (Roy et al., 2013) estimated a recent split between the Hawaiian mints and their closest NW *Stachys* relatives (~3.6–4.7 Mya), followed by rapid migration, colonization and diversification into the Hawaiian Islands. In most cases, branch lengths from individual gene trees can provide information on the relative time intervals between divergence events (Holder et al., 2001). However, in our individual gene trees the branches at nodes where two different homeologs of the Hawaiian taxa diverge from their closely related parental taxa do not appear to be shallower compared to the rest of the tree (Fig. 2A–E), suggesting rapid diversification among not only the Hawaiian taxa but also the NW *Stachys* taxa. Consequently, if the incongruent patterns due to the polyphyletic

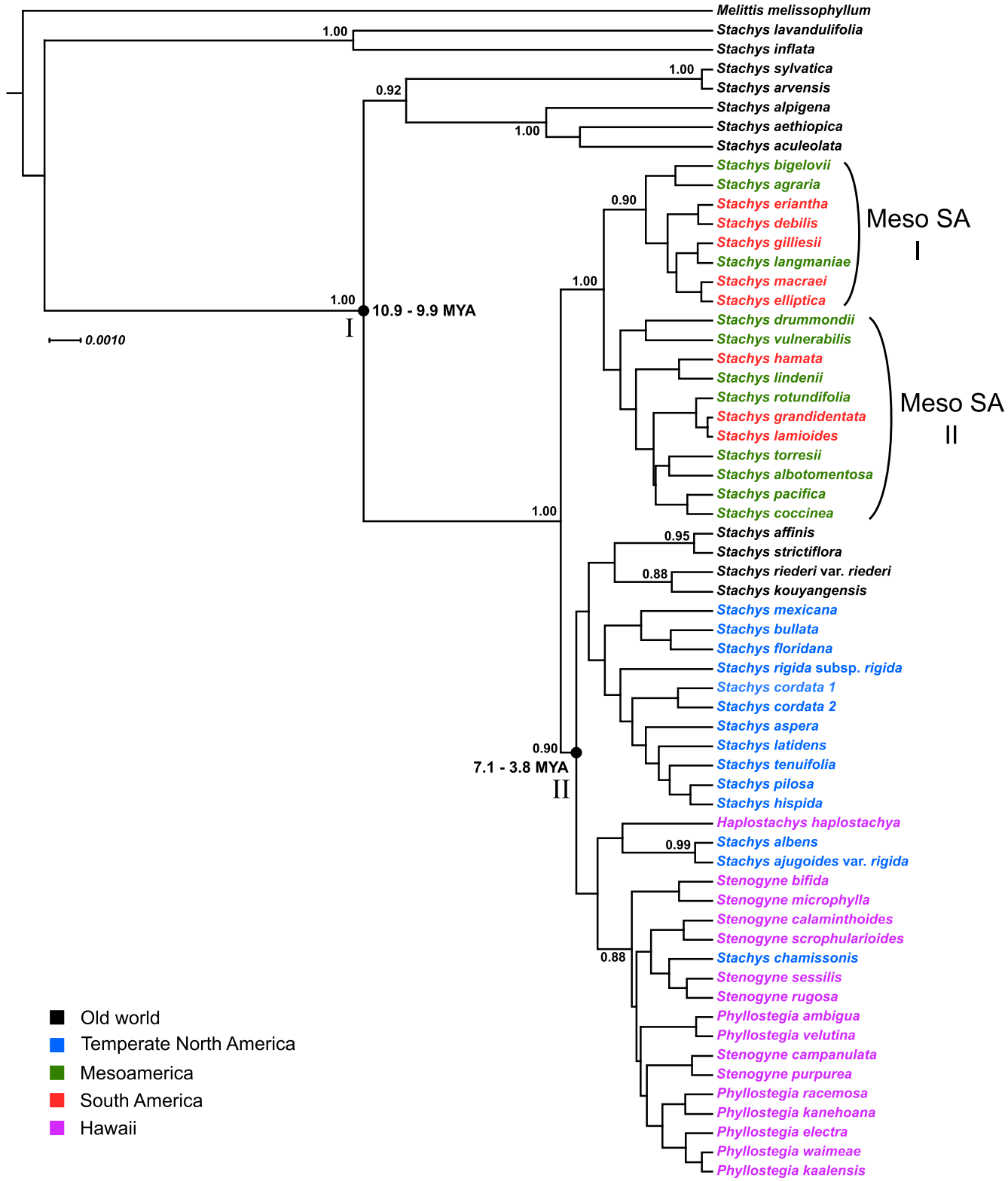

Fig. 3. Multi-locus coalescent tree ("species tree") as inferred from the *BEAST analysis. Numbers at the nodes indicate posterior probability values (PP). Only those nodes with PP values above 0.85 have been labeled. Numbers following taxon names refer to the different accessions for *S. cordata*. Meso-SA = Meso-South America. Taxa have been color coded according to their geographic locality (see key). Results obtained for our divergence time analysis from BEAST for three loci (*WAXY*, *ADK* and *COR*) have been mapped on this tree. Mean divergence times estimates are shown at the nodes. Refer to Table 3 for 95% HPD (highest posterior density) values.

placements of the Hawaiian mints, observed from our individual gene trees, resulted solely from the incomplete sorting of ancestral polymorphisms (because of insufficient time interval between their divergences), we would have expected the NW *Stachys* to be polyphyletic as well, with intermingling between Meso-SA and temperate NA species. However, this is not the scenario observed. Hence, the persistence of ancestral alleles was probably initially introduced into the population as a result of inter-specific

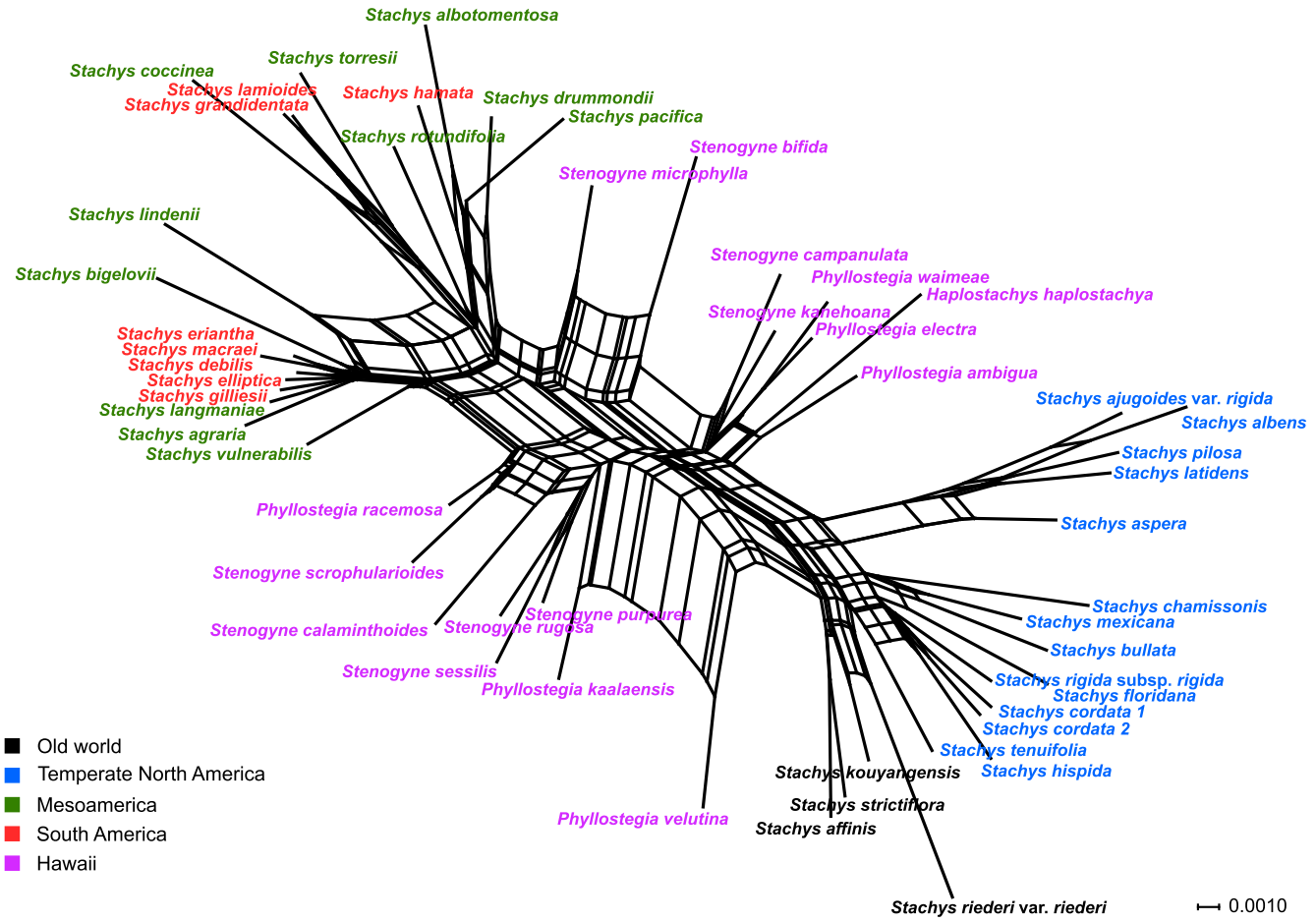

Fig. 4. Neighbor-Net network for the concatenated dataset from the five loci. Numbers following taxon names refer to the different accessions for *S. cordata*. Taxa have been color coded according to their geographic locality (see key).

Table 3
Estimated node ages for selected divergence events under a relaxed clock model using the *ADK*, *WAXY* and *COR* datasets. Ages are in million years with the 95% HPD (highest posterior density) within brackets.

Node of interest	*WAXY*	*ADK*	*COR*
I	10.1 (6.6–13.4)	9.9 (6.8–13.2)	10.9 (8.2–13.2)
II	6.0 (3.3–9.6)	3.8 (2.3–5.7)	7.1 (4.6–9.7)

gene flow between the ancestors of the Hawaiian mints during sympatry, although ILS cannot be completely discounted as an additional factor leading to their continued existence within the population.

Incongruence due to hybridization and gene flow is expected to be confined to specific nodes among the gene trees, whereas ILS is expected to generate random sets of discordant gene trees (Howarth and Baum, 2005). In four of our five nuclear gene trees (*WAXY*, *ADK*, *COR* and *AFO*; Fig. 2A–D and Table S2), Hawaiian mints consistently group with Meso-SA I and temperate NA *Stachys* members, with no occurrence of them grouping within the Meso-SA II clade. The only exception to this being the *NIA* locus where the two Meso-SA clades remained unresolved (Fig. 2E; also see Section 4.4). This leads us to assume a possible hybrid ancestry of the Hawaiian taxa, involving the above mentioned two *Stachys* lineages. Although we do not observe further resolution of relationships between the Hawaiian mints and their Meso-SA I and temperate NA *Stachys* relatives, our deductions are also led by the fact that in case of *WAXY*, *ADK*, *COR* and *NIA*, each of these clades are robustly supported by Bayesian posterior probability values (Fig. 2A, B, C, and E respectively), and for *AFO* (Fig. 2D), strong support is observed in case of the clade comprising temperate NA *Stachys* and the Hawaiian species.

The Bayesian tree for the *AFO* locus (Fig. 2D), followed by *ADK* and *COR* (Fig. 2B and C, respectively), shows the greatest number of Hawaiian taxa with homeologs present in both clades. This observed grouping of homeologs of the Hawaiian taxa could have resulted from differential loss of homeologs following hybridization (Wendel, 2000). Kim et al. (2008) noted such groupings of multiple copies of different *Persicaria* (Polygonaceae) taxa in different clades and postulated hybridization to be one of the major causes. This could have also arisen from differential sampling of clones from the different loci (Supplementary Table S1), from which only one parental homeolog of the hybridization event may have been randomly selected. An increased sampling of clones could have resulted in homeologs from Hawaiian species grouping into both clades for all the loci under investigation. It is worth mentioning that within putative hybrid species, where lineage sorting is complete, we expect the homeologs to be monophyletic, grouping with either one of the parental species for different genes. We observe this trend for all the Hawaiian taxa in our *WAXY* tree (Fig. 2A), and for certain species in the trees for the other three loci (*ADK*, *COR* and *AFO*; Fig. 2B–D; Table S2).

Our observations from the coalescent-based multi-locus analyses ("species trees"; Fig. 3 and S1) show incongruence with the individual gene trees, since the Hawaiian taxa here group with the temperate NA *Stachys* species only. The coalescent model as implemented in *BEAST and STRAW assumes incomplete lineage sorting as the main source of topological incongruence, with the

absence of any gene flow. It has been shown in a recent study by Leaché et al. (2013) that paraphyletic (between non-sister species) and ancestral gene flow both play an important role leading to gene tree-species tree discordance, and might result in generating a species tree, which obfuscates true species relationships. Hence, since estimation of our species trees is based explicitly on multi-locus coalescence models assuming only ILS, ancestral and paraphyletic gene flow from hybridization between the putative parental species (temperate NA and Meso-SA *Stachys*) of the Hawaiian lineage is likely a major cause leading to this discordance between the species trees and gene trees. As a result of this gene flow the species trees recovered in our study may not be accurately reflecting the true topology, which is probably better delineated in our individual gene trees, resolving the Hawaiian mints as polyphyletic, with their different homeologues grouping with the different putative parents. The observed similarity between our current species trees and the nrDNA trees from our previous study (Roy et al., 2013), may be caused by biased concerted evolution and gene conversion. Hence, the nrDNA phylogenies may reflect the phylogenetic history inherited from only one parent (likely the paternal one), since they showed an incongruent placement of the Hawaiian mints as compared to the maternally inherited cpDNA phylogeny. This previous cytonuclear discordance was also hypothesized to be a result of a hybrid origin of the Hawaiian taxa (Roy et al., 2013).

In summary, a number of consistent patterns seen here provide support to our hypothesis that the Hawaiian mints had a possible origin from hybridization events between members of two distinct lineages of *Stachys* species, the temperate NA and Meso-SA I clades, rather than being a result of ILS alone: (1) polyphyly among the Hawaiian endemic mints, but not among the NW *Stachys*, with Hawaiian mint homeologs nested within two distinct *Stachys* clades, (2) the intermediate position of the Hawaiian mints in the Neighbor-Net analysis (Fig. 4) mirrored by significant amount of reticulation between the Hawaiian mints and the temperate NA and Meso-SA clades, respectively, (3) the multi-locus coalescent species trees are in discordance with the individual gene trees, and (4) previous results based on cpDNA and nrDNA data, where the Hawaiian mints group with either Meso-SA *Stachys* or temperate NA *Stachys*, the latter topology mirrored by the species trees. It is also important to note that the presumably limited population sizes of island species, such as the Hawaiian mints, do not allow the prevalence of ILS, which usually has a greater effect on large ancestral populations. In such cases, the major causes for deep coalescence and the prevalence of ancestral alleles in large populations is a lower rate of genetic drift (Edwards, 2009).

4.2. Origin of the Hawaiian mints: tracing the putative NW Stachys parents

Hybridization has played an important role in the phylogenetic relationships among plants undergoing reticulate evolution (Soltis and Soltis, 2009; Abbott et al., 2013). The flora of the Hawaiian Islands has been noted to have the highest incidence of polyploidy known, with most Hawaiian species probably being paleopolyploids, where polyploidization evolved in their ancestors prior to their migration to and colonization of the Hawaiian Islands (Carr, 1998; Baldwin and Wagner, 2010). As discussed in Section 4.1, the polyploid Hawaiian mints seem to have arisen from hybridization involving two separate *Stachys* lineages, which might have resulted in low sequence divergence but high intra-individual polymorphisms (Lindqvist and Albert, 2002; Lindqvist et al., 2003, 2006, 2007; Roy et al., 2013). It is possible that polyploidization in the ancestors of the Hawaiian mints, and possibly other Hawaiian lineages, was a result of hybridization followed by an increase in ploidy through the union of unreduced gametes from the two parental species. Such novel genetic combinations in the genomes of the polyploid hybrid progeny could have lead to innovative genetic changes affecting their colonization abilities, allowing them to occupy new environmental niches, and giving rise to a diversity of new species (Soltis and Soltis, 2000; Mallet, 2007; Bento et al., 2008; Van de Peer et al., 2009).

Our current data, as well as previous observations (Lindqvist and Albert, 2002; Roy et al., 2013), suggest that the Hawaiian mints originated from interbreeding between sympatric ancestors of temperate NA and Meso-SA I parents. Initial allopolyploidization could have taken place through hybridization between ancestral parental taxa with chromosome numbers of $2n = 32$ (Meso-SA I) and $2n = 34$ (temperate NA), giving rise to temperate western NA *Stachys* species and Hawaiian mints ancestors with similar chromosome numbers ($2n = 64$ and 66). For example, temperate western NA *S. chamissonis* ($2n = 64$) is nested with the Hawaiian mints in the majority of our gene trees (*COR*, *AFO* and *NIA* loci; Fig. 2C, D, and E, respectively) and the species trees (Figs. 3 and S1), and *S. mexicana* ($2n = 66$) shows a similar phylogenetic placement in the *WAXY* and *AFO* gene trees (Fig. 2A and D, respectively). Other temperate western NA close relatives are *S. albens* ($2n = 66$) and *S. ajugoides* var. *rigida* ($2n = 66$), which together with *S. chamissonis* are nested inside the Hawaiian mint clade in the *BEAST species tree. All these species are distributed predominantly in the southwestern US (*S. albens*, *S. ajugoides* var. *rigida*), or throughout western US (*S. chamissonis*), pointing toward a probable origin of the Hawaiian endemic mint ancestor in southwestern US, where the hybridization events may have occurred prior to colonization of the Hawaiian Islands. These findings corroborate previous results by Lindqvist and Albert (2002) and Roy et al. (2013), the latter study suggesting the matrilineal lineage of the Hawaiian mints to have originated in the Meso-SA region. Since most of the Hawaiian mints sampled to date are recorded to have a chromosome number of $2n = 64$ (Carr, 1998), it is likely that the ancestral species colonizing the Hawaiian Islands also was $2n = 64$, such as a close relative of *S. chamissonis*. However, it has been hypothesized that *Stachys* species with chromosome numbers $2n = 66$ and 64 derived from ancestors with the base number of $x = 17$ ($2n = 68$) through chromosomal fusion (Mulligan and Munro, 1989). It is possible that such chromosomal fusion took place in the Hawaiian Islands as well, giving rise to the $2n = 64$ species. Improved phylogenetic resolution and additional chromosome counts would be needed to test this hypothesis. Nevertheless, an allopolyploid western US *Stachys* relative may have been the progenitor of the extant Hawaiian mints, migrating to the Hawaiian Islands via a single colonization event, which was also suggested by Roy and Colleagues (2013) and Lindqvist and Albert (2002). The probability that the Hawaiian Islands was colonized only once is supported by the monophyletic position of the Hawaiian mints in these previous studies, grouping with the temperate NA *Stachys* in nrDNA and Meso-SA *Stachys* in the cpDNA phylogenies. Although our current data show polyphyletic positions of the Hawaiian mints, this is likely caused by placements of homeologs in both parental lineages. Differential loss of homeologs following hybridization, and possibly differential sampling of clones, may have resulted in conflicting positions of individual Hawaiian mint species in different gene trees (see also Sections 4.1 and 4.4).

Their diverse morphological and ecological phenotypes could also indicate a hybrid ancestry for the Hawaiian lineage. For example, members of Meso-SA I clade are characterized by small, purple-white flowers, whereas members of temperate western NA *Stachys* have variable floral morphologies ranging from smaller white flowers (less than 12 mm in length) in *S. albens* and *S. ajugoides*, to large bright pink-purple flowers (corolla tube 15–28 mm long) in *S. chamissonis* and *S. mexicana* (Mulligan and Munro, 1989). The Hawaiian mints exhibit a wide range of

morphological and floral diversity, ranging from mostly white to pink corollas in *Haplostachys* (21–25 mm in length) and *Phyllostegia* (up to 25 mm in length), to the predominantly pink-red corollas in *Stenogyne* (up to 56 mm in length) (Lindqvist et al., 2003), clearly indicating a reticulate origin from parental lineages bearing a variety of floral morphologies as well as diverse habitats (ranging from moist habitats in coastal areas and marshy plains in species like *Stachys chamissonis*, *S. ajugoides* and *S. albens* to species like *S. eriantha* and *S. radicans* distributed in low-mid elevation montane regions; Turner, 1994). An allopolyploid hybrid ancestry of the Hawaiian mints could have allowed them to adapt to and occupy new habitats, thereby radiating and diversifying across different habitats resulting in a wide range of phenotypic variation. Baldwin and Sanderson (1998) pointed to a similar hybrid ancestry of the Hawaiian silversword alliance (*Argyroxiphium*, *Dubautia*, *Wilkesia*: Asteraceae) from North American tarweeds (*Madia*, *Raillardiopsis*: Asteraceae) leading to their wide range of ecological and morphological diversity in the islands. Such reticulate evolution has also been observed in Hawaiian violets (*Viola*: Violaceae; Marcussen et al., 2012). Romaschenko and Colleagues (2014) studied ancient hybridization events in *Patis* and *Ptilagrostis* (Poaceae: Stipeae) and showed how deep reticulation and introgressive hybridization played an important role in the evolution of grasses. They concluded that *Ptilagrostis* is of ancient hybrid origin with its nuclear genome retained from an early or proto-*Ptilagrostis* precursor with possible origins in North America.

4.3. Tracing the evolutionary relationships among the Meso-South American Stachys

The *Stachys* species from southwestern United States, Mexico, and South America group into two well-supported clades (Meso-SA I and II) in gene trees based on *ADK*, *WAXY* and *COR* loci (Fig. 2A–C; Table S3), as well as the species trees (Figs. 3, S1; Table S3), and the nrDNA phylogenies (Roy et al., 2013). These two clades show similar species compositions to their nrDNA phylogenies, especially the 5S-NTS gene tree, which was better resolved than the ETS tree (Roy et al., 2013). In the *NIA* tree (Fig. 2E), all Meso-SA species group into one clade, and in the *AFO* tree (Fig. 2D) only the Meso-SA II clade is resolved, whereas other species loosely resembling Meso-SA I clade members remain unresolved and interspersed with the Hawaiian mints. The SA *Stachys* species remain nested within the two Meso-SA clades (Figs. 2A–E and 3 and Table S3). Most SA members of the Meso-SA I clade (*S. macraei*, *S. eriantha*, *S. gilliesii*, and *S. sericea*) have small purple-white flowers (corolla length varying between 5 and 11 mm). The corolla lengths of the SA members of Meso-SA clade II range between 2 and 15 mm, e.g., *S. grandidentata* exhibits flowers 5–7 mm long, whereas *S. lamioides* possesses larger flowers ranging between 10 and 15 mm. This is in general conjunction with the floral compositions of the Mesoamerican members of these clades, indicating floral similarities between the SA taxa and their putative Mesoamerican relatives. The Mesoamerican members of Meso-SA clade I are mostly characterized by species with small leaves and small cream-purple flowers (corolla length varying between 2 and 12 mm), whereas members within Meso-SA clade II, including the *Stachys coccinea* complex, bear large orange-red, annulate corollas (12–25 mm long; Turner, 1994). Four of the five members of the *S. coccinea* species complex (*S. torresii*, *S. coccinea*, *S. albotomentosa* and *S. pacifica*) are monophyletic, although not supported in the species tree (Fig. 3), showing their close relationships within the Meso-SA II lineage. Some Meso-SA taxa from clade I (*S. agraria*, *S. drummondii*, *S. biflora*, *S. eriantha*, and *S. gilliesii*) and *S. coccinea* from clade II possess similar pollen morphologies (tectate-perforate apocolpia; Bassett and Munro, 1986), suggesting some introgression between the two Meso-SA clades. Such introgression is also supported by clones of *S. lindenii*, *S. drummondii*, *S. coccinea*, and *S. pacifica* grouping in both Meso-SA I and II clades (Table S3). It is possible the endemic SA *Stachys* could have arisen via introgressive hybridization between the two clades of Meso-SA *Stachys* species serving as the putative parents. The mixed phylogenetic signals from the incompatible splits between the two clades of Meso-SA taxa in our network analysis (represented by the multiple connections between branches; Fig. 4) also reflect a reticulate origin for the SA *Stachys*. Similar to the Hawaiian lineage, also considering the recent age of the South American taxa (Roy et al., 2013), alternative explanations include incomplete sorting of ancestral polymorphisms and gene duplication followed by retention of paralogous gene copies. For example, *S. torresii* and *S. coccinea* clones cluster with both the Meso-SA II clade and in a separate clade with tropical African *Stachys* in *AFO* (Fig. 2D).

4.4. Evolutionary history of NIA for NW Stachydeae

Similarly to the other gene trees, the *NIA* tree (Fig. 2E) groups the temperate NA and the Meso-SA taxa in separate clades. However, the *NIA* gene tree shows a discordant pattern from the other gene trees, as well as the species tree, exhibiting only a single large clade of Meso-SA taxa including the Hawaiian mints, whereas the temperate NA taxa form two separate clades. As per Mulligan and Munro's cytotaxonomic studies (1989), *Stachys* species distributed North of Mexico, consist of a mix of both functional diploids and tetraploids (e.g., *Stachys latidens* and *S. tenuifolia* have populations of $2n = 34$ and 68) as well as hexaploids (e.g., *S. pilosa* with $2n = 102$). The base chromosome number for the temperate NA *Stachys* is $x = 17$. Autopolyploidization ($2n = 34 \rightarrow 68$; $2n = 32 \rightarrow 64$) as well as the suggested allopolyploidization (see Section 4.2) in NW Stachydeae could have resulted in a variety of cardinal genomic changes including non-random elimination of coding and non-coding DNA sequences, epigenetic changes such as DNA methylation of coding and non-coding DNA leading to gene silencing and activation of genes and retro-elements, which in turn alters the expression of adjacent genes. These effects have been investigated at length in a number of reviews (Leitch and Bennett, 1997; Comai, 2000; Soltis and Soltis, 2000; Wendel, 2000; Pikaard, 2001), and evolution of low-copy nuclear genes in polyploids has provided valuable insights into the origins of polyploid genomes in recent studies (e.g., Brysting et al., 2007; Kim et al., 2008).

It is possible that the radically different results from our *NIA* tree is the result of elimination of low-copy DNA sequences from homoeologous copies of the chromosomes in some of the taxa, leading them to form separate clades in the temperate NA *Stachys*, and causing the Meso-SA members to group into one big polytomized clade. Liu et al. (1998) found such elimination of non-coding, low-copy DNA sequences, occurring in all the diploid species of the genera *Aegilops* and *Triticum* (Poaceae) but only in one pair of chromosomes (chromosome-specific sequences, CSSs), or in several chromosome pairs of one genome (genome-specific sequences, GSSs) in allopolyploid wheat. Intergenomic translocations are frequently observed in polyploids and suggest that historic recombination between homeologous chromosomes may be a common occurrence (e.g., Zwierzykowski et al., 1998). Interlocus interactions have not been extensively studied in single- or low-copy genes, due to the difficulties associated with isolating proven orthologs and homeologs between diploid ancestors whose phylogenetic relationships could be studied and their derived polyploid descendants (Wendel, 2000). Previous studies have suggested that evolutionary success and greater survival of polyploids have been mostly attributed to the large number of different gametic combinations generated by independent

assortment combined with inter-genomic exchanges (Zohary and Feldman, 1962). It is also worth mentioning that the genomes of many naturally occurring diploids also harbor signatures of past cryptic hybridizations and polyploid ancestry (Cronn et al., 1996), which might be the case in putative diploid NW *Stachys* species.

4.5. Divergence timings and historical biogeography of the New World Stachydeae

Our results suggest that Stachydeae members initially diversified into the NW during the Middle-Late Miocene period (Fig. 3 and Table 3) when they split from their closest OW (Mediterranean/African) relatives. Similar to results from cpDNA and nrDNA data, some OW East Asian species are also embedded within the temperate NA *Stachys* clade based on analysis of the low-copy nuclear loci, indicating a second bout of colonization to temperate NA during the early Pliocene period (Lindqvist and Albert, 2002; Roy et al., 2013). Previous geological and paleobotanical studies have suggested that the Beringian Land Bridge (BLB) interconnecting Eurasia and North America acted as an important highway for temperate elements during the Neogene/Paleogene era (Wen, 1999; Tiffney, 2008; Tiffney and Manchester, 2001). Marincovich and Gladenkov (1999) have postulated the minimum age range for the opening of the Bering Strait to be between 4.8 and 7.4 Mya. According to our divergence timings, dispersal across the Beringian Land Bridge seems to be one of the plausible migratory routes of the NW Stachydeae lineage during the Late Miocene period. However, long distance dispersal across the Atlantic Ocean for this first colonization event is equally likely (Roy et al., 2013). Similar East Asian/Eurasian and temperate NA geographical disjunctions have been observed in many other angiosperm lineages, including another mint group, subtribe Nepetineae (Lamiaceae; Drew and Sytsma, 2013). Due to a paucity of resolution within our current gene trees, we were unable to shed further light onto the timing of migration and colonization events of the Hawaiian mints.

5. Conclusion

Utilizing independently evolving low-copy nuclear loci, we have been able to reconstruct and shed further light onto the origin and evolutionary relationships among the NW and Hawaiian Stachydeae members. Our results corroborate our previous findings (Lindqvist and Albert, 2002; Lindqvist et al., 2003, 2006, 2007; Roy et al., 2013) that were based on chloroplast and nuclear ribosomal DNA sequence data. Hence, we have revisited the historical biogeography of the NW Stachydeae, tracing their divergence into the Americas followed by rapid cladogenesis. We also now have data from different genomic compartments that convey a scenario for the Hawaiian endemic mint lineage that is best explained by a hybrid, possibly allopolyploid, ancestry from members of separate lineages of temperate North American and Meso-SA *Stachys*. As such, this study provides insights into polyploid hybridization and rapid diversification in both continental and oceanic insular settings. Evidence of the importance of polyploidy in angiosperm diversification may come from such studies of recent radiations in young, unstable environments, such as the volcanic Hawaiian Islands (Soltis et al., 2009) or Andean South America (Tank and Olmstead, 2009). For example, dispersal to volcanic islands often involve colonization of newly formed habitats that over relatively short time change dramatically with geological erosions and island subsidence, and this process repeats itself as new islands form. Interestingly, the Hawaiian mints radiated and diversified to a great extent following their polyploid hybrid origin and migration to new habitats, flourishing in their newly carved niches. Polyploidization and hybridization usually provides combinations of new genotypes leading to an increased potential to adaptation in new habitats in the offspring as compared to their progenitors species (Mable, 2013). This in turn facilitates speciation and diversification in novel environments and may have been important in the establishment and evolutionary success of many Hawaiian plant species (Baldwin and Wagner, 2010). It is possible that early angiosperms may have experienced similar environmental instabilities before their successful establishment worldwide (Soltis et al., 2009). On the other hand, the Hawaiian mints' closest extant relatives, possibly of a similar hybrid origin, on the American continent did not appear to experience such rapid diversification in their more established, mainland habitats (e.g., *Stachys chamissonis* and *S. mexicana*).

Similar to other island endemics, many Hawaiian mint species are extinct, rare, or endangered today, and taxonomic knowledge on the majority of the species in this lineage comes from collections in herbaria. Together with previous studies of nuclear ribosomal and plastid DNA sequence data (Lindqvist and Albert, 2002; Lindqvist et al., 2003, 2006, 2007; Roy et al., 2013), these new data based on five nuclear loci provide one of the most comprehensive datasets based largely on herbarium material from a remarkable Hawaiian plant radiation. However, further studies with multiple populations from each species, and possibly a phylogenomic approach, will be helpful in providing additional insight into the roles of hybridization and introgression toward the origin of the endemic Hawaiian mints, as well as the South American endemic *Stachys*.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2015.03.023.

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